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CHELATION OF IRON BY SUGARS

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SUMMARY

Reducing sugars and polyols are shown to form soluble stable complexes with a series of metal ions at alkaline pH. Several properties of an iron–fructose complex have been studied. The specific conditions of pH and concentrations of iron and fructose necessary for complex formation are described. Evidence for the existence of the complex is (a) its solubility at alkaline pH, (b) its characteristic spectral properties, (c) titration and redox measurements, and (d) the direct isolation of the water-soluble complex.

Ferric–fructose can be isolated and purified by precipitation from an aqueous solution with ethanol or other organic solvents. Elemental analysis indicates the complex at pH 9.0 is formed with 2 Fe: 2 fructose: 1 Na. Ferric–fructose is a low-molecular-weight compound which rapidly dialyses through a Visking sac. It is isoionic at pH 4.5–4.7. Metal–sugar complexes may play an important biological role in the transport of the mineral elements across cell membranes.

INTRODUCTION

One of the interesting aspects of the control and regulation of the metabolism of trace ions is the passive nature of this process[1]. Whereas the transport of bulk cations, such as K^+, Na^+, Ca^{2+}, and Mg^{2+} seems directly coupled to metabolic energy and accumulation is by active transport, the trace metal ions, including Fe^{3+}, Cu^{2+}, and Mn^{2+} are not necessarily intimately linked to respiratory energy[2]. Recently we have shown that the transport of iron into liver cells from the β_1-globulin, transferrin, is facilitated by low-molecular-weight chelates of the iron[3]. The kinetics of this transport are regulated by the equilibrium binding of the iron to the various low- and high-molecular-weight sites available in the system. In the search for sequestering agents of iron which would be useful in the elucidation of the role of chelates in the transport of trace metals, we have discovered that fructose and other sugars, under specific circumstances, can form highly stable complexes with metal ions. These chelates have unique chemical properties which make them particularly interesting for their role in biological systems.

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Sugar complexes have been previously reported. However, these have been either insoluble or slightly soluble[4–6], or high-molecular-weight colloidal preparations[7–9]. Soluble calcium complexes with saccharate or levulate have been used in the purification of sugar cane juice and fructose, respectively[10]. Recently MILLS[11] has demonstrated the formation of soluble calcium complexes with a variety of polyols.

METHODS AND RESULTS

The preparation of iron–sugar chelates in this research was carried out as follows: solutions of reagent-grade $FeCl_3$, $Fe(NO_3)_3$, $FeCl_2$, and $FeSO_4$ were prepared with distilled water. Where a standard iron preparation was required to provide comparative data over a period of several days, precipitation of the hydroxide was prevented by the addition of concentrated HNO_3 to bring the pH below 1. Sugar solutions of the desired molarity were prepared in distilled water. Since some of the sugars have appreciable negative heats of solution, the mixture was permitted to reach room temperature before use. The sugar and iron solutions were then mixed and the pH adjusted to the desired value with 1 N or 6 N NaOH.

Spectral evidence for chelate formation

The color of dilute $Fe(NO_3)_3$ solution is normally a pale yellow at acid pH. Upon the addition of excess sugar, the color darkens considerably, with a slight decrease in pH. Such a change in spectral properties has been used to establish the presence of a chelate[12]. It was discovered that equimolar proportions of iron to monosaccharide were completely ineffectual in preventing hydroxide precipitation under alkaline conditions, although the spectral change was apparent.

The color of the ferric–fructose complexes is dark yellow under acid conditions, red-brown under alkaline conditions. The hydroxylated ferric ion absorption is very strong in the lower wavelengths so that the presence of the darker complex, which is apparent visually, cannot easily be demonstrated with the spectrophotometer. The spectral properties of this ferric–fructose chelate can be observed by measuring the difference spectrum between a 10^{-3} M $Fe(NO_3)_3$ solution at pH 2.5 and the same concentration of $Fe(NO_3)_3$ in the presence of a 16-fold molar excess of fructose at the same pH (Fig. 1).

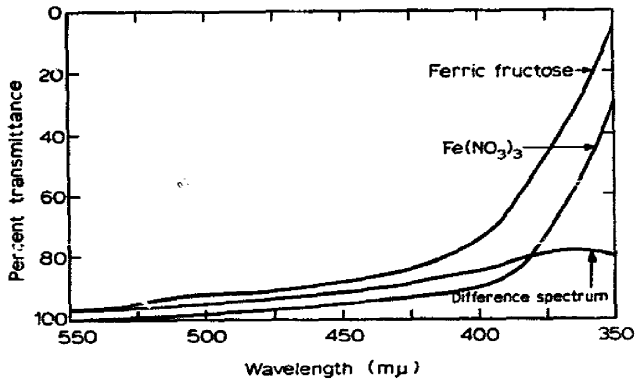


Fig. 1. Difference spectrum of ferric–fructose compared to ferric ion. All solutions were adjusted to pH 2.5.

The increased absorption of this complex at 370 mμ is clearly demonstrable, despite the close similarity of the spectra for the individual solutions.

Titrimetric and potentiometric evidence for complex formation

The displacement of one or more hydrogen ions from the sugar molecule during the formation of a fructose chelate was directly demonstrated by the titration curves shown in Fig. 2. 50 ml of a solution 0.25 M fructose, 0.01 N HCl, and 0.012 N in either Fe^{3+} or Fe^{2+} was titrated with 0.11 N NaOH. In order to be certain that no interaction of Fe^{2+} and Fe^{3+} would interfere with the titration, 50 ml of a third solution 0.25 M fructose, 0.01 N HCl, 0.006 N Fe^{3+} and 0.006 N Fe^{2+} was likewise titrated. The pH was determined with a Radiometer Model-22 pH meter. The titration curves reveal that 3 H^+ are directly displaced from the sugar and the H_2O for each Fe^{3+} initially present. Complex formation is complete for Fe^{3+} by pH 3.5. In the study with Fe^{2+}, 2 H^+ are displaced per iron. However, complex formation does not begin until about pH 7.5 and is essentially complete by pH 8.5. At all times both solutions were clear. No precipitate or colloid formation could be detected. The titration curve for Fe^{3+} and Fe^{2+} in the same solution indicates that there is no interference between the oxidized and the reduced iron ions.

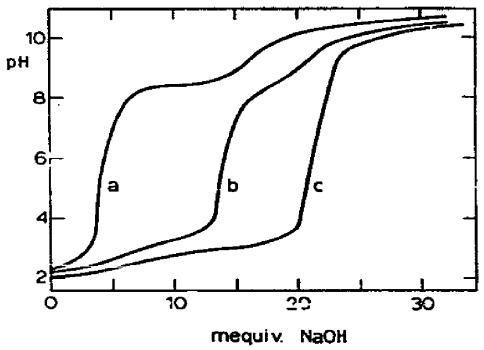


Fig. 2. Titration curves of Fe^{3+} and Fe^{2+} separately and together in the presence of excess fructose. The initial volume in each case was 50 ml of a solution 0.25 M fructose, 0.01 N HCl, and (a) 0.012 M Fe^{2+}, (b) 0.006 M Fe^{2+} and 0.006 Fe^{3+}, or (c) 0.012 M Fe^{3+}

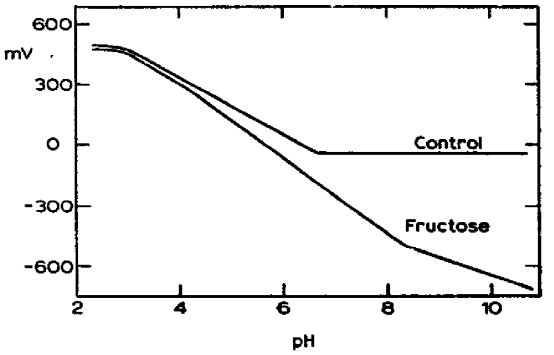


Fig. 3. Redox potential as a function of pH with and without fructose. Initial solution was 0.006 M Fe^{3+} and 0.006 Fe^{2+}; fructose, when present, 0.25 M.

Potential measurements over a wide range of pH were made in a solution containing 0.006 M Fe^{3+} and 0.006 M Fe^{2+}. The pH and redox potential were measured simultaneously using two Model-22 Radiometers, one fitted with a glass electrode, the other with a platinum electrode, using a standard calomel half cell. The initial pH of the solution was 2.0. It was rapidly titrated with 0.11 N NaOH. The values obtained are presented in Fig. 3. However, when the same measurements are made in the presence of 0.25 M fructose it can be seen in Fig. 3 that the Fe^{3+} and Fe^{2+} are in equilibrium over a range which extends to pH 10.5. The break in the slope of the line at pH 8.25 is caused by the formation of the Fe^{2+} complex with the concomitant lowering of the concentration of this free ion in solution. The slope of the redox potential *vs.* pH line of Fig. 3 is that which one would find if 3 H^+ were liberated in

the chelation of Fe^{3+} by fructose, and confirms the titrimetric evidence presented above. This system is not freely reversible and hysteresis effects were observed. Therefore only qualitative conclusions can be obtained.

Ability of other sugars and polyols to form complexes with iron

In preliminary experiments it was noted that an excess of sugar over iron is required in order to form stable soluble chelates at alkaline pH. An evaluation of the relative sequestering abilities of several reducing sugars and polyols was obtained as follows: solutions 0.1 M in ferric iron were prepared containing increasing concentrations of the sugar being tested, and these solutions were made alkaline, as previously described. The minimum concentration of sugar adequate to prevent the precipitation of a hydroxide was taken as an index of its sequestering ability. The relative sequestering ability of polyols and sugars for trivalent iron was found to be: fructose > sorbitol > glucose = galactose = maltose = lactose > sucrose > ribose > erythrose.

Relative iron and fructose concentrations necessary for complex formation

During the course of these experiments it became apparent that not only was the ratio of iron to sugar, but also the absolute concentrations of the two, of critical importance for chelation. An investigation into these relationships resulted in the data presented in Fig. 4. Each point represents a solution containing iron and fructose at the concentrations indicated on the coordinates. Each solution was titrated with 6 N NaOH from its original pH of 2, through neutral and alkaline conditions to pH 12. The turbidity of the solutions was observed visually.

Fructose can sequester iron at concentrations twice that of the metal, but only when the concentration of the sugar is near saturation. The region around pH 7, where precipitation occurs with low sugar concentration, is of considerable interest. Evidence has been presented above that a complex is formed immediately upon the addition of iron to the carbohydrate. As a solution of such a complex is made basic, the complex will remain in solution beyond the pH where the uncomplexed metal ion would otherwise precipitate as the hydroxide. If there is an insufficient amount of

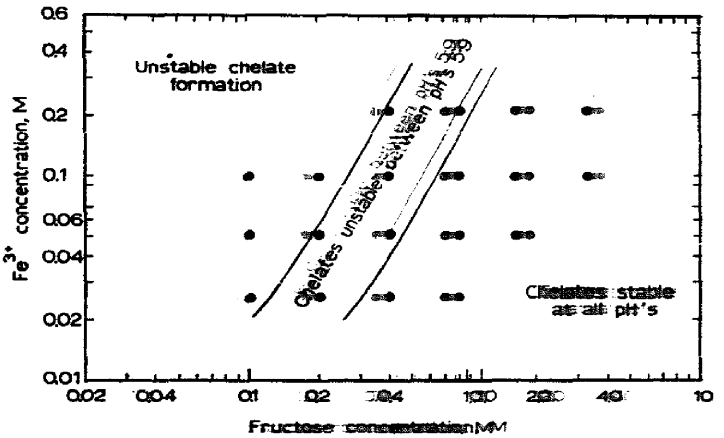


Fig. 4. Solubility of ferric–fructose complex as a function of the concentration of Fe^{3+} and fructose.

carbohydrate present, a precipitate will occur as the solution becomes alkaline. Above pH 8 the precipitate will redissolve and remain in solution up to or past pH 14.

The ability of fructose to form complexes with other metal ions

Solutions were prepared containing 1.0 M fructose and 0.01 M of the following: Fe^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Ni^{2+}, and Co^{2+}. The initially acid solutions were gradually increased to pH 12 with 1.0 N NaOH. The absence of a colloidal precipitate in the presence of sugar, as compared to controls with no sugar, was taken as qualitative evidence for the formation of a complex. Only Cd^{2+} did not form a chelate.

Formation of ferrous–fructose complexes

Complexes of Fe^{2+} with fructose can be prepared, as indicated above, but only at carbohydrate:iron ratios approximately double those required for successful sequestering of Fe^{3+}. The characteristic color of the complexes of both Fe^{3+} and Fe^{2+} reflect the presence of the hydroxylated iron. Ferric–fructose is a deep red-brown, while ferrous–fructose is blue-green. Solutions of ferric–fructose in the presence of excess fructose gradually turn to a gray-brown color over a period of several weeks. Tests of such solutions with dipyridyl revealed that the Fe^{3+} was gradually converted to Fe^{2+}. The characteristic pink color of the ferrous–dipyridyl complex developed more rapidly in acid than in alkaline solution. It is known that oxidation of sugars by metal ions proceeds more rapidly in alkaline solution[13]. The apparently anomalous redox behavior of the ferric–fructose could be caused by the tighter binding of the metal at high pH, thus removing it from effective participation. There was no appreciable photoxidation under normal laboratory lighting.

Ionophoretic behavior of the iron complexes

Paper-ionophoresis experiments were conducted using Whatman No. 3 filter-paper strips and 0.1 M buffer at an applied current of 2.5 mA, applied voltage 450 V. The location of the iron chelates was detected by spraying the air-dried paper strips with acid ferrocyanide. The position of the fructose band was identified with a resorcinol spray[14]. Since there is buffer movement by electro-osmosis, the free fructose zone was taken as the origin to establish the distance which the chelates had migrated. Ferric–fructose was prepared using 0.1 M $FeCl_3$ and 3.2 M fructose, and ferric–citrate was prepared by using 0.1 M $FeCl_3$ and 0.2 M citric acid. Stable soluble chelates of iron with malic and tartaric acid were prepared from solutions containing 0.2 M organic acids and 0.1 M $FeCl_3$. The chelate solutions were adjusted rapidly to the desired pH with 1 N NaOH immediately prior to use, mixed with an equal volume of the buffer, and 0.01 ml applied to the paper strip.

At all pH values tested, the tartrate and malate chelates migrated at the same rate as the citrate chelates. By ionophoresis at various pH's near the pK_a's of the three buffer systems, acetate, formate and bicarbonate, it was determined that the isoelectric region of ferric–fructose is between 4.5 and 4.7. The ferric complex of citrate is still negatively charged at this pH as shown by Fig. 5.

Isolation and characterization of the ferric–fructose complex

The ferric–fructose complex can be precipitated from aqueous solution by the

addition of ethanol to a final concentration of 80% (v/v). The complex was prepared by rapidly titrating 20.0 ml of a solution 0.1 M in $FeCl_3$ and 1.0 M in fructose to pH 9.0, 80 ml of absolute ethanol were added, the pH checked again, and the precipitate formed collected by centrifugation. The precipitate was redissolved in a small volume of water, the pH adjusted again, and this clear solution again made 80% in ethanol. The precipitate formed was collected by centrifugation, washed with

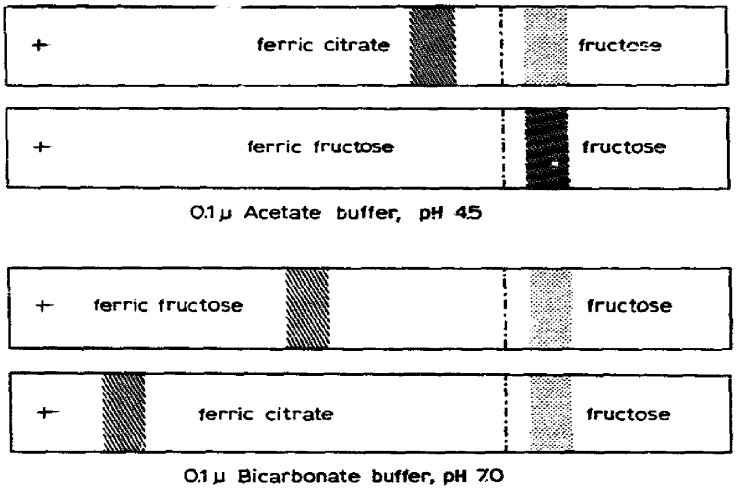


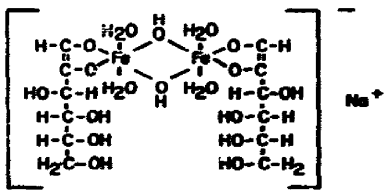
Fig. 5. Electrophoresis patterns of ferric chelates at pH 4.5 and 7.0.

TABLE I

ANALYSIS BY WEIGHT PER CENT OF THE ELEMENTAL COMPOSITION OF FERRIC–FRUCTOSE ISOLATED AT pH 9.0 AND OF A PROPOSED STRUCTURE OF SUCH A COMPLEX

	C	*H*	*O*	*Fe*	*Na*	H_2O^*
Ferric-fructose	26.1	3.6	43.5	22.9	4.0	10.5
Proposed structure	27.5	4.2	42.8	21.4	4.2	12.1

* H_2O removed by drying in high vacuum. The oxygen value given does not include that from the water of hydration.



successive volumes of ethanol, acetone, and ether, and dried over P_2O_5. Elemental analysis for C, H, Fe, Na and O, by difference, was performed after thorough drying in high vacuum*. These values are presented in Table I with the calculated weight

* Mikroanalytisches Laboratorium im Max Planck Institut für Kohlenforschung, Mulheim, and Laboratorium Fresenius, Wiesbaden (Germany).

per cent values of a hypothetical model for the complex. Since ferric–fructose is insoluble in the organic materials commonly used for molecular-weight determinations by freezing-point lowering, an aqueous solution, containing 50.3 mg/ml of the material used in the elemental analysis, was prepared and its freezing-point lowering measured as 0.315°. If one assumes complete dissociation of the Na salt of the complex, the experimental value for the mol. wt. is 594. The mol. wt. calculated for the proposed structure is 597.

DISCUSSION

We have presented evidence that sugars and polyols, under specific conditions of concentration and of pH, form stable and soluble complexes with a wide variety of metal ions which would otherwise be insoluble or sparingly soluble in an alkaline medium. Large molar excesses of sugar to metal ion favor chelate formation. This finding explains the results of BOURNE *et al.*[6], who studied complex formation with excess metal ions added to small concentrations of polyol and sugar. It is interesting to note that the complexes they investigated contained molar ratios of metal to chelating agent which varied from 0.35 to 10.75. Their inability to show complex formation of iron with glucose, and of cobalt and nickel with dulcitol or glucose, can be attributed to both the ratios of metal to sugar present and to their technique of adding the metal salts to a pH-12 solution of the chelating agent where the competition of hydroxide ion will prevent the successful sequestering of iron by most ligands for this metal.

It is very difficult to characterize the precise chemical and physical properties of the fructose chelates. Preliminary experiments employing spectrophotometric techniques indicate a value of about 10^{-2} for the association constant of Fe^{3+} for the first hydroxyl group of fructose. Although chelation and complex formation of trace metals with alcohols and polyols is often alluded to in the literature, few, if any, definitive binding constants are to be found. The chelation of iron by gluconic acid has been studied[15], but there is some uncertainty with respect to the molecular configuration of the complex and the interpretation of the titration data[16]. Much more precise measurements employing techniques of electron spin and nuclear magnetic resonance, magnetic susceptibility, crystal structure, etc. are needed. Preliminary spin-resonance studies suggest that the two iron atoms are interacting and thus reducing the magnitude of the signal. The copper–fructose, on the other hand, gives a well defined, specific spectrum.

We have shown that these sugar chelates are effective in facilitating the diffusion of iron across the membrane of the intestine mucosal wall[17]. It has long been known that dietary carbohydrates enhance the accumulation of iron in experimental animals[18]. Pathological findings in such animals mimic in many ways those observed in the condition of Bantu siderosis[19]. The diet of the Bantu consists primarily of a corn gruel, prepared by extensive cooking in cast iron pots. Such a diet offers conditions favorable for the formation of iron complexes with carbohydrates, when the food enters the small intestine from the acid environment of the stomach. The feeding of the iron–fructose chelate does, indeed, enhance the deposition of iron in the spleen and liver of rats.

The realization that sugars or polyols form stable soluble complexes with metal ions permits greater understanding of the mechanism whereby metal ion uptake from

the diet is stimulated by such compounds. HERNDON *et al.*[20] demonstrated that iron uptake was enhanced in rats in the presence of sorbitol. Since sorbitol cannot reduce the iron to the ferrous form, these authors postulated that the polyol either stimulated the absorption by an unknown mechanism within the mucosal wall, or was active by protecting various substances in the intestinal lumen. FOURNIER *et al.*[21] and WASSERMAN *et al.*[22] showed that several carbohydrates, as well as certain amino acids, enhance calcium uptake from the rat gut. LENGEMANN[23] has recently extended these experiments and demonstrated that preloading the animal with carbohydrates, either by feeding or by intubation, does not stimulate calcium uptake. Lactose or glucose, the two sugars tested, must be present concomitantly with the metal ion in the intestine for maximum stimulation.

The effectiveness of the chelate to facilitate metal-ion transport is directly related to the net charge and molecular size. LARSEN *et al.*[24] have demonstrated that the EDTA chelate of iron is poorly absorbed by rats, despite its excellent solubility and stability characteristics. The EDTA complex carries a net negative charge in the range of pH encountered in the gastrointestinal tract, except possibly in the stomach and immediately distal to the pyloric valve. High-molecular-weight carbohydrate complexes, such as saccharated oxide of iron or iron–dextran, are poorly absorbed from the intestine, whereas low-molecular-weight complexes of iron with fructose and other simple sugars described above readily penetrate biological membranes such as encountered in the mucosal wall.

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REFERENCES

1 P. SALTMAN, *Proc. United Nations 2nd. Intern. Conf., Peaceful Uses of Atomic Energy, Geneva*, Vol. 24, 1958, p. 152.
2 G. C. COTZIAS, *Physiol. Revs.*, 38 (1958) 503.
3 P. J. CHARLEY, M. ROSENSTEIN, E. SHORE AND P. SALTMAN, *Arch. Biochem. Biophys.*, 88 (1960) 222.
4 W. TRAUBE, F. KUHBIER AND H. HARTING, *Ber.*, 66 (1933) 1945.
5 T. LEISER AND R. EBERT, *Ann. N.Y. Acad. Sci. U.S.*, 532 (1937) 89.
6 E. J. BOURNE, R. NERY AND H. WEIGER, *Chemistry and Industry*, (1959) 998.
7 C. G. B. SLACK AND J. F. WILKINSON, *Lancet*, 1 (1949) 11.
8 J. A. NISSIN AND J. M. ROBSON, *Lancet*, 1 (1949) 686.
9 T. BERSIN, *Pharm. Acta Helv.*, 26 (1951) 407.
10 P. HONIG, *Principles of Sugar Technology*, Elsevier, Amsterdam, 1955, p. 5.
11 J. A. MILLS, *Biochem. Biophys. Research Communs.*, 6 (1961) 418.

[12] S. CHABEREK AND A. E. MARTELL, *Organic Sequestering Agents*, Wiley, New York, 1959, p. 43.
[13] E. WERTHEIM, *Textbook of Organic Chemistry*, Blakiston, New York, 1945, p. 265.
[14] S. ARONOFF, *Techniques of Radiobiochemistry*, Iowa State College Press, Ames, 1956, p. 102.
[15] R. L. PECSOK AND J. SANDERA, *J. Am. Chem. Soc.*, 77 (1955) 489.
[16] S. CHABEREK AND A. E. MARTELL, *Organic Sequestering Agents*, Wiley, New York, 1959, p. 314.
[17] C. STITT, P. CHARLEY, P. SALTMAN AND E. HOTT, *Proc. Soc. Exptl. Biol. Med.*, 110 (1962) 70.
[18] T. D. KINNEY, D. M. HEGSTED AND C. A. FINCH, *J. Exptl. Med.*, 90 (1949) 137.
[19] J. GILLMAN AND T. GILLMAN, *Arch. Pathol.*, 40 (1945) 239.
[20] J. F. HERNDON, E. G. RICE, H. G. TUCKER, E. J. VAN LOON AND S. M. GREENBERG, *J. Nutrition*, 64 (1958) 615.
[21] P. FOURNIER, Y. DUPUIS, H. SUBBIELLE, M. ALLEAND AND N. TARDY, *J. Physiol., (Paris)* 47 (1955) 339.
[22] R. H. WASSERMAN, C. L. COMAR AND M. M. NOLD, *J. Nutrition*, 59 (1956) 371.
[23] F. W. LENGEMANN, *J. Nutrition*, 69 (1959) 233.
[24] B. A. LARSEN, R. G. S. BIDWELL AND W. W. HAWKINS, *Can. J. Biochem. Physiol.*, 38 (1960) 51.